Acid-triggered release of doxorubicin from a hydrazone-linked Gd^{3+}-texaphyrin conjugate

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Contents

1. Synthetic materials and methods

All reagents were purchased from Fisher Scientific, Aldrich, or TCI and used without further purification. All solvents were analytical or HPLC grade. Deionized water was used unless otherwise indicated. Organic solvents were purified using a solvent purifier system (Vacuum Atmospheres) unless otherwise indicated. Dichloromethane was freshly distilled after being dried over CaH_2 under argon. Reverse-phase HPLC experiments were conducted using a Shimadzu HPLC (Shimadzu LC 6AD) with a Thermo Scientific Acclain™ 120 C18 (3 μm, 120 Å, 2.1 x 150 mm) Shim-pack GIS (5 μm ODS, 250 x 4.6 mm id) column for analytical studies and Waters Sep-Pak® Vac 35 cc (10 g) *t*-C18 Cartridges for preparative work. The flow rates for the analytical studies was 1.0 mL/min. For the mobile phase, Buffer A (water with 0.1% v/v acetic acid) and Buffer B (acetonitrile with 0.1% v/v acetic acid) were used to provide the solvent gradient. Mass spectrometric analyses were carried out in the University of Texas at Austin Mass Spectrometry Facility. Low-resolution and high-resolution electrospray mass spectrometric (ESI-MS) analyses were carried out using a Thermo Finnigan LTQ instrument and a Qq-FTICR (7 Telsa) instrument, respectively.

2. UV/Vis and fluorescence spectroscopic methods

All organic solvents used for spectroscopic analyses were HPLC grade free of fluorescent impurities. Stock solutions of conjugate **1** were prepared in DMSO. Phosphate buffered saline (PBS) (20 mM, pH 7.4) and acetate buffer (20 mM, pH 5.0) were prepared in deionized water. All spectra were recorded in this buffer solution containing 1% (v/v) DMSO. The fluorescence and UV/Vis absorption spectra were recorded on Shimadzu RF-5301PC and S-3100 spectrophotometers, respectively. Excitation was carried out at 500 nm with both the excitation and emission slits widths being set at 3 nm.

3. T_1-weighted MR contrast properties of conjugate 1 in PBS solution

The T_1 relaxivity of conjugate **1** in aqueous solution was measured so as to evaluate its potential for MR imaging. Conjugate **1** was analyzed at various concentrations in PBS solution at 60 MHz (1.4 T) and 200 MHz (4.7 T) using a Minispec system (Bruker, Germany), and a MRI (Biospec 47/40, Bruker, Germany) system, respectively, and imaged using a 4.7 T MRI instrument (Biospec 47/40). The following scanning parameters were used: MSME (Multi-slice multi-echo) pulse sequence, TE/TR = 9.4/350 ms, matrix size = 192 × 192, FOV = 2 × 6 cm, slice thickness = 1 mm. Linear fitting of the measured relaxation rates ($R_1 = 1/T_1$, s^{-1}) vs. the Gd^{3+} concentration (mM) was used to determine the relaxivity values, r_1.

4. Cell culture and fluorescence imaging

Human lung cancer A549, mouse colon carcinoma CT26, and mouse fibroblast NIH3T3 cells were grown in 5% CO_2 at 37°C in Dulbecco's modified Eagle's medium (DMEM) supplemented with 1 × Antibiotic-Antimycotic (Invitrogen-Gibco, Carlsbad, CA, USA) and 10% fetal bovine serum (Invitrogen-Gibco). For fluorescence microscope imaging, the cells were seeded on 8-well μ-slides (Ibidi, Munich, Germany) for 24 h and were treated with **1** at a concentration of 4 μM for an additional 1 h or 12 h. The cells were then washed 3 times and fixed in Cytofix fixation buffer (BD Biosciences, San Jose, CA, USA) for 30 min at 4°C and counterstained with Hoechst 33342 (Molecular Probes, Eugene, OR, USA) at 5 μg/mL. For co-localization studies, CT26 cells were stained with 100 nM LysoTracker Green DND-26 (Molecular Probes) or 50 nM MitoTracker Green FM (Molecular Probes) for 1 h at 37°C. Fluorescence images were obtained using a laser scanning confocal microscope (LSM 710, Carl Zeiss, Germany).

5. Cell viability measurement of cells treated with conjugate 1

The cell viability of A549, CT26, and NIH3T3 cells incubated with conjugate **1** was assessed by a 3-(4,5-dimethylthiazole-2-yl)-2,5-diphenyl tetrazolium bromide (MTT; Roche Diagnostics GmbH, Mannheim, Germany) assay. The cells were seeded in 96-well plates for 24 h at a density of 2×10^3 cells/well and

then replaced with fresh culture medium. The cells were treated with various concentrations of **1**, and incubated for 48 h. MTT in a solubilizing buffer as purchased commercially (Roche Diagnostics) added to each well and allowed to incubate for 4 h. Absorbance was measured at 570 nm using a microplate reader (VersaMax; Molecular Devices, CA, USA).

6. T_1-weighted MR contrast enhancement of cells treated with conjugate 1

T_1-weighted spin-echo images of cell phantoms were obtained on a 4.7 T MRI instrument using A549 and CT26 cells labeled with different concentrations of **1**. The following acquisition parameters were used: Field of view (FOV) = 5.0 × 2.5 cm^2 and 4 × 5 cm^2, matrix size = 192 × 192, slice thickness = 1 mm, echo time (TE) = 9.4 ms, repetition time (TR) = 350 ms.

7. Synthesis of compounds 1-3

The starting texaphyrin (**MGd**) was obtained as a gift from Pharmacyclics, Inc. or reprepared using literature procedures.[1,2]

Synthesis of compound 3: **MGd** (300 mg, 0.26 mmol) and 4-nitrophenyl chloroformate (525 mg, 2.61 mmol) were dissolved in distilled dichloromethane (DCM) (60 mL). *N,N*-diisopropylethylamine (DIPEA) (930 μL, 5.22 mmol) was slowly added to the reaction mixture, which was then stirred for 4 h at room temperature. The progress of the reaction was monitored by HPLC. When the starting material (**MGd**) was no longer present (as inferred from this analysis), the solvent was evaporated off under reduced pressure and the resulting precipitates were collected with the aid of diethyl ether washings. The product, **3**, was obtained as a dark green solid in 87% yield (330 mg). ESI-MS m/z $[M\text{-}2OAc]^{2+}$ calc. 680.20900,

1 J. L. Sessler, N. A. Tvermoes, D. M. Guldi, T. D. Mody and W. E. Allen, *J. Phys. Chem. A*, 1999, **103**, 787.
2 W.-H. Wei, M. Fountain, D. Magda, Z. Wang, P. Lecane, M. Mesfin, D. Miles and J. L. Sessler, *Org. Biomol. Chem.,* 2005, **3**, 3290.

obs. 680.21060.

Synthesis of compound 2: To a solution of **3** (200 mg, 0.13 mmol) in acetonitrile (CH_3CN) (10 mL), hydrazine monohydrate (68 µL, 1.35 mmol) was slowly added. The reaction mixture was then stirred for 2 h at room temperature. The progress of the reaction was monitored by HPLC. When the starting material **3** was no longer present (as inferred from this analysis), the solvent was evaporated off under reduced pressure and the resulting residue was purified using Waters Sep-Pak® Vac 35 cc (10 g) tC18 Cartridges. For this purification, 50 mL of a 0.1 M ammonium acetate buffer solution (4 L of distilled water containing 32 g of ammonium acetate and 40 mL of acetic acid) was added and the resulting solution was loaded onto the C18 cartridge and subject to elution with an increasing gradient of CH_3CN (10~90%) in an ammonium acetate buffer. The product, **2**, eluted off when the eluent contained 25~30% of CH_3CN. The fraction obtained in this way was then loaded on a new C18 cartridge, desalted with HPLC-grade deionized water, and eluted off using pure methanol (MeOH). The volatiles were removed under vacuum to give the product **2** as a dark green solid in 30% yield (50 mg). ESI-MS m/z $[M\text{-}2OAc]^{2+}$ calc. 573.21930, obs. 573.22090.

Synthesis of compound 1: Doxorubicin·HCl (20 mg, 0.016 mmol) and **2** (28 mg, 0.047 mmol) were dissolved in anhydrous methanol (MeOH) (10 mL) and the mixture was treated with trifluoroacetic acid (TFA) (5 µL). After stirring for 2 days at room temperature in the dark, all volatiles were evaporated off under vacuum. The residue obtained in this way was purified using Waters Sep-Pak® Vac 35 cc (10 g) tC18 Cartridges. Here, 50 mL of a 0.1 M ammonium acetate buffer solution (4 L of distilled water containing 32 g of ammonium acetate and 40 mL of acetic acid) was added and the resulting solution was loaded onto the C18 cartridge and subject to elution with increasing gradient of CH_3CN (10~90%) in an ammonium acetate buffer. The product, **1,** eluted off when the eluent contained 20~30% of CH_3CN. The

fraction obtained in this way was then loaded on a new C18 cartridge, desalted with HPLC-grade deionized water, and eluted off using pure methanol (MeOH). The solvent was removed under vacuum to give product **1** as a brown solid in 51% yield (19 mg). ESI-MS m/z $[M\text{-}2OAc]^{2+}$ calc. 1,098.38850, obs. 1,098.38730.

8. Additional experimental data

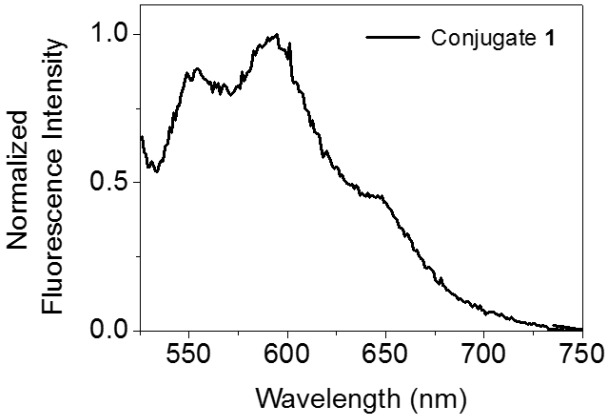


Figure S1. Normalized fluorescence spectrum of conjugate **1** in PBS buffer (pH 7.4) at 37°C with an excitation wavelength at 500 nm.

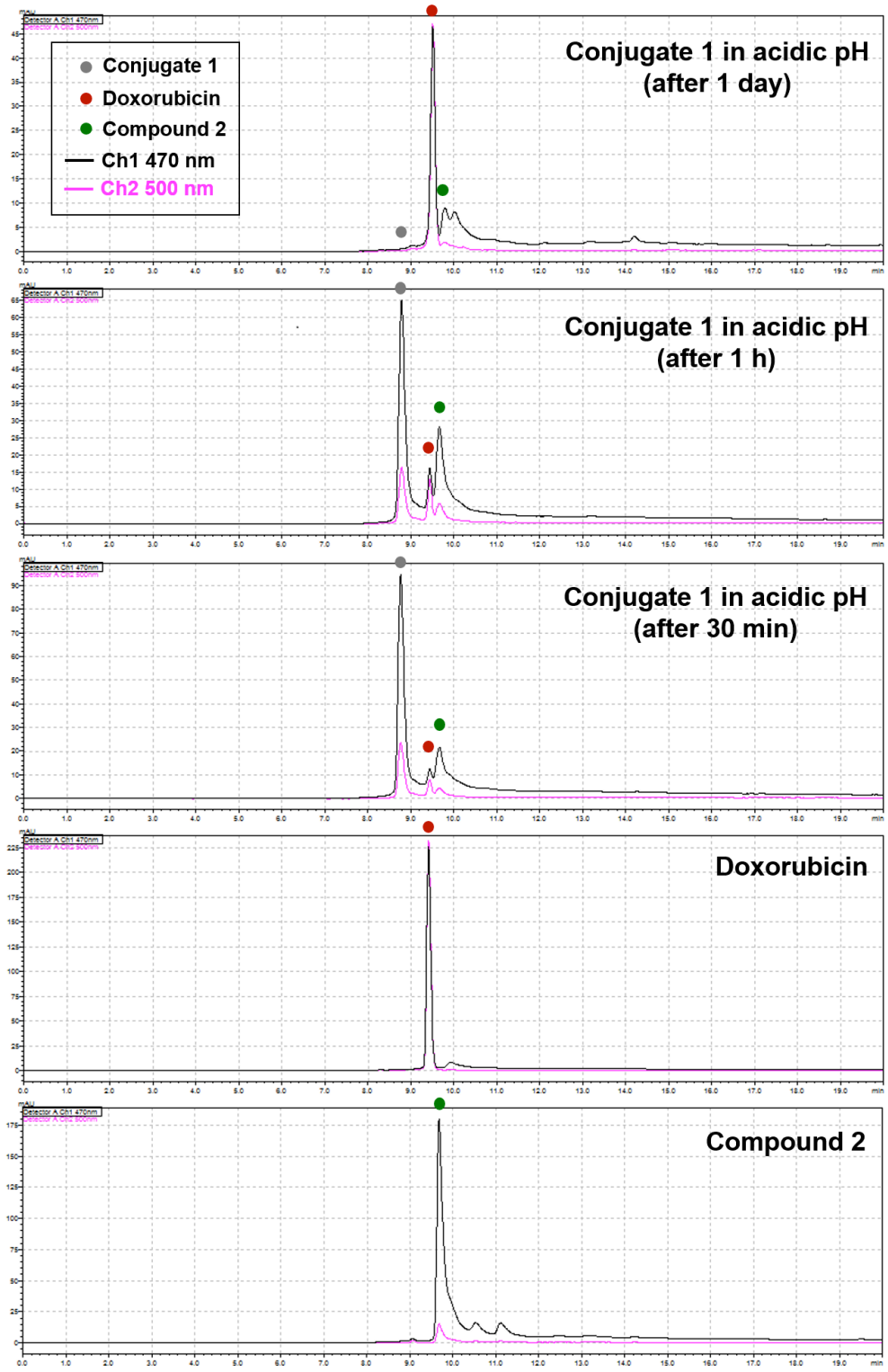


Figure S2. HPLC chromatograms of conjugate **1** at an acidic pH (acetate buffer; pH 5.0), **doxorubicin**, and **2**. Peaks in the chromatograms were detected by monitoring the UV/Vis absorption at 500 (pink) and 470 nm (black), respectively.

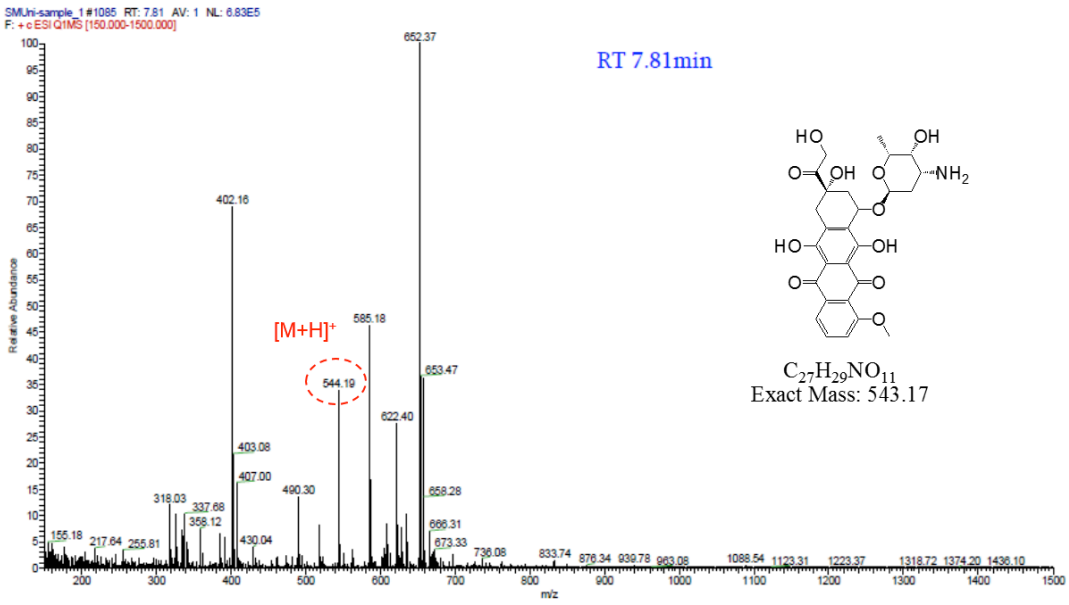


Figure S3. ESI-Mass spectrum for the **Dox** released from conjugate **1** when allowed to sit in an acetate buffer at pH 5.0.

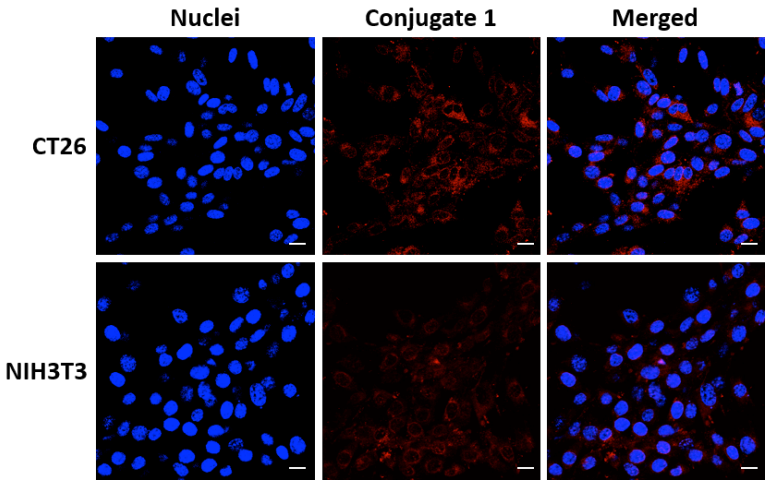


Figure S4. Fluorescence images of cells treated with conjugate **1**. CT26 and NIH3T3 cells were treated with 4 μM of **1** for 12 h. The cells were then fixed in 4% paraformaldehyde after washing with PBS and then stained with Hoechst (nuclear counterstain, blue). Images were obtained using excitation wavelengths of 405 nm and 543 nm, with the emission being monitored over the 420–480 nm and 560–615 nm spectral regions for the blue and red signals, respectively. Scale bar: 20 μm.

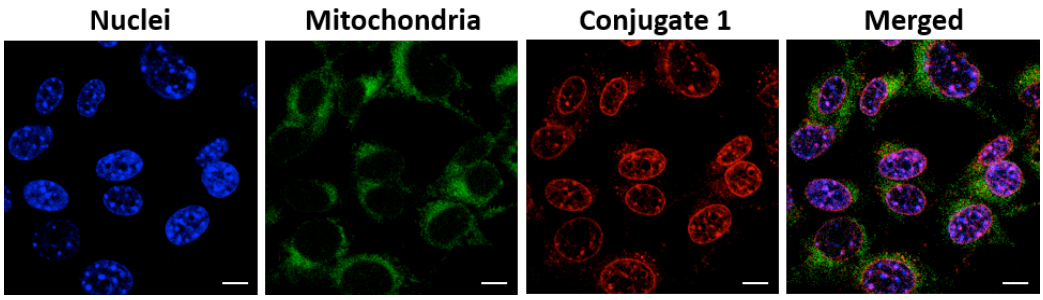


Figure S5. Fluorescence images of CT26 cells treated with Hoechst (blue), MitoTracker (green), and conjugate **1** (red). Cells were treated with 10 μM of conjugate **1** for 12 h. Cells were then fixed in 4% paraformaldehyde after washing in PBS and stained with Hoechst (blue) and MitoTracker (green). Scale bar: 10 μm.

9. HPLC chromatograms and ESI-MS data for compounds 1-3

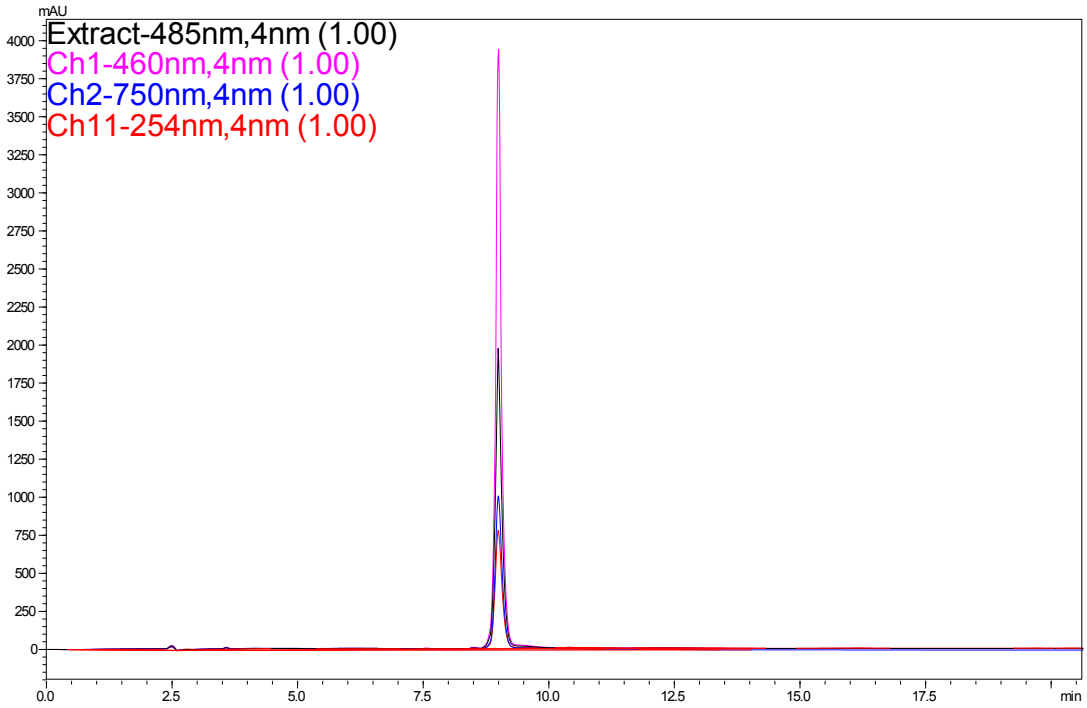
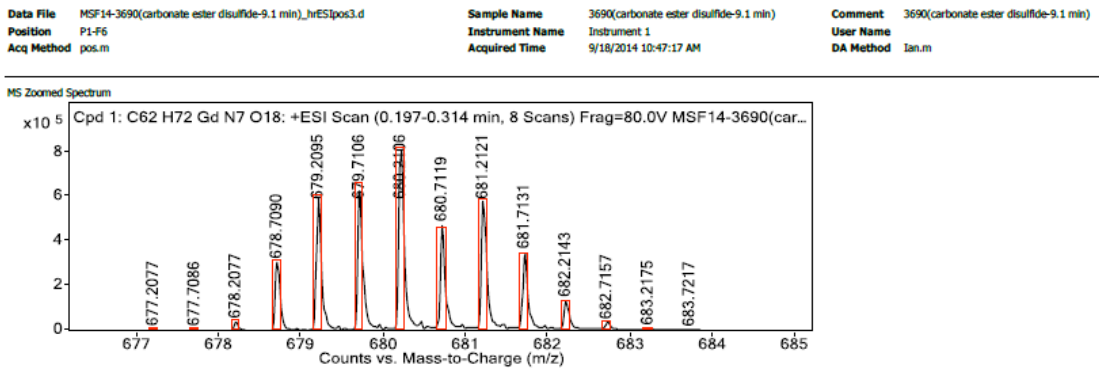


Figure S6. HPLC chromatogram of **3**.



MS Spectrum Peak List

Obs. m/z	Calc. m/z	Charge	Abund	Formula	Ion/Isotope	Tgt Mass Error (ppm)
677.20770	677.20600	2	3878.57	C62H72GdN7O18	M+2	-2.47
678.20770	678.20660	2	37916.52	C62H72GdN7O18	M+2	-1.58
678.70900	678.70750	2	306265.47	C62H72GdN7O18	M+2	-2.21
679.20950	679.20790	2	595161.31	C62H72GdN7O18	M+2	-2.36
679.71060	679.70870	2	649825.16	C62H72GdN7O18	M+2	-2.78
680.21060	680.20900	2	811379.91	C62H72GdN7O18	M+2	-2.43
680.71190	680.71020	2	473737.78	C62H72GdN7O18	M+2	-2.51
681.21210	681.21020	2	588835.02	C62H72GdN7O18	M+2	-2.74
681.71310	681.71150	2	351113.53	C62H72GdN7O18	M+2	-2.37
682.21430	682.21280	2	134305.2	C62H72GdN7O18	M+2	-2.16

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Figure S7. ESI-MS spectrum of **3**.

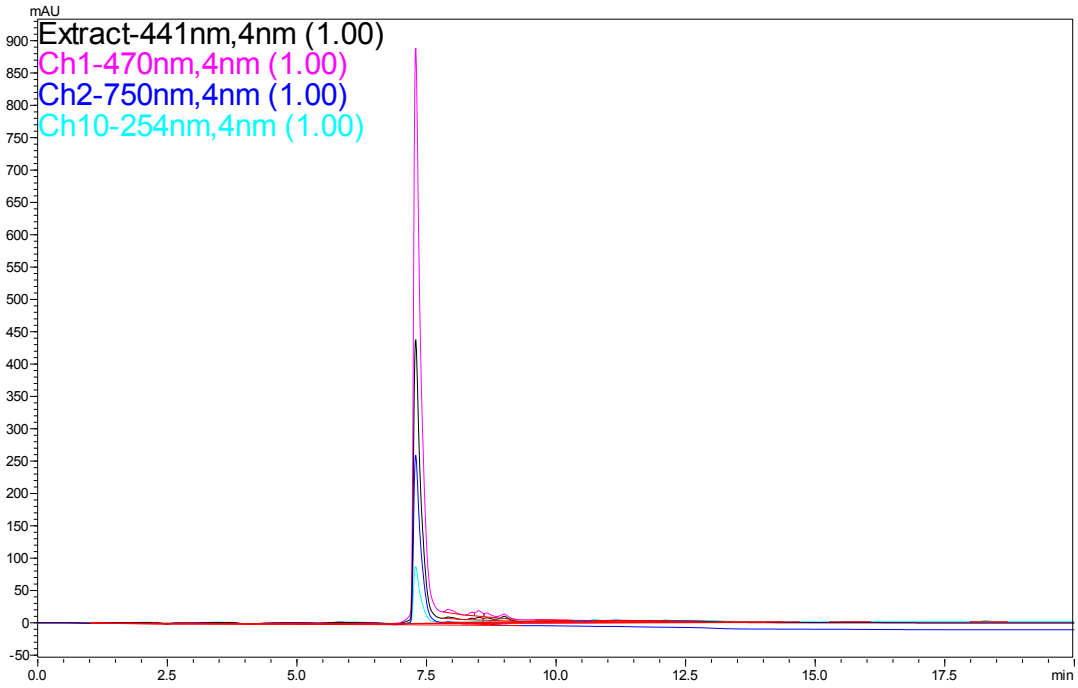
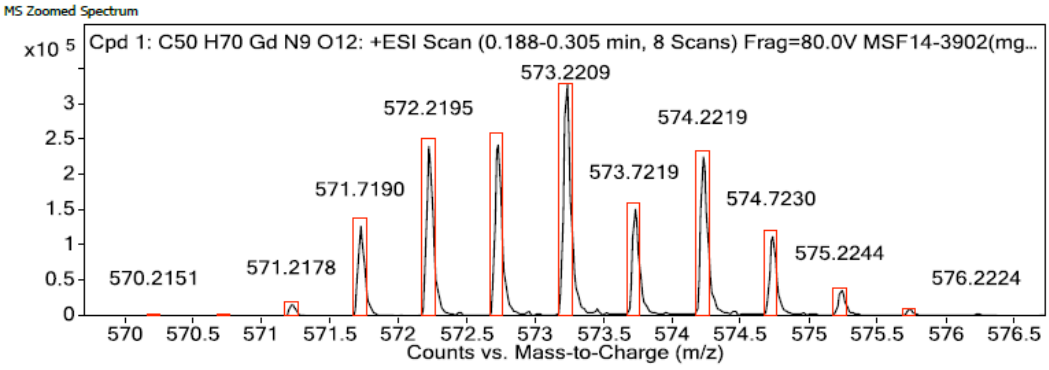


Figure S8. HPLC chromatogram of **2**.

Data File	MSF14-3902(mgd dihydrazine)_hrESIpos2.d	**Sample Name**	3902(mgd dihydrazine)	**Comment**	3902(mgd dihydrazine)
Position	P1-D1	**Instrument Name**	Instrument 1	**User Name**	
Acq Method	pos.m	**Acquired Time**	9/23/2014 2:10:48 PM	**DA Method**	Ian.m



MS Spectrum Peak List

Obs. m/z	Calc. m/z	Charge	Abund	Formula	Ion/Isotope	Tgt Mass Error (ppm)
570.21510	570.21650	2	2345.57	C50H70GdN9O12	M+2	2.46
571.21780	571.21710	2	17398.97	C50H70GdN9O12	M+2	-1.25
571.71900	571.71800	2	128692.05	C50H70GdN9O12	M+2	-1.76
572.21950	572.21830	2	244050.76	C50H70GdN9O12	M+2	-2.19
572.72040	572.71910	2	248020.64	C50H70GdN9O12	M+2	-2.26
573.22090	573.21930	2	328237.87	C50H70GdN9O12	M+2	-2.78
573.72190	573.72060	2	153147.36	C50H70GdN9O12	M+2	-2.31
574.22190	574.22050	2	231096.19	C50H70GdN9O12	M+2	-2.42
574.72300	574.72180	2	115272.09	C50H70GdN9O12	M+2	-2.01
575.22440	575.22320	2	37989.15	C50H70GdN9O12	M+2	-2.17

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Figure S9. ESI-MS spectrum of **2**.

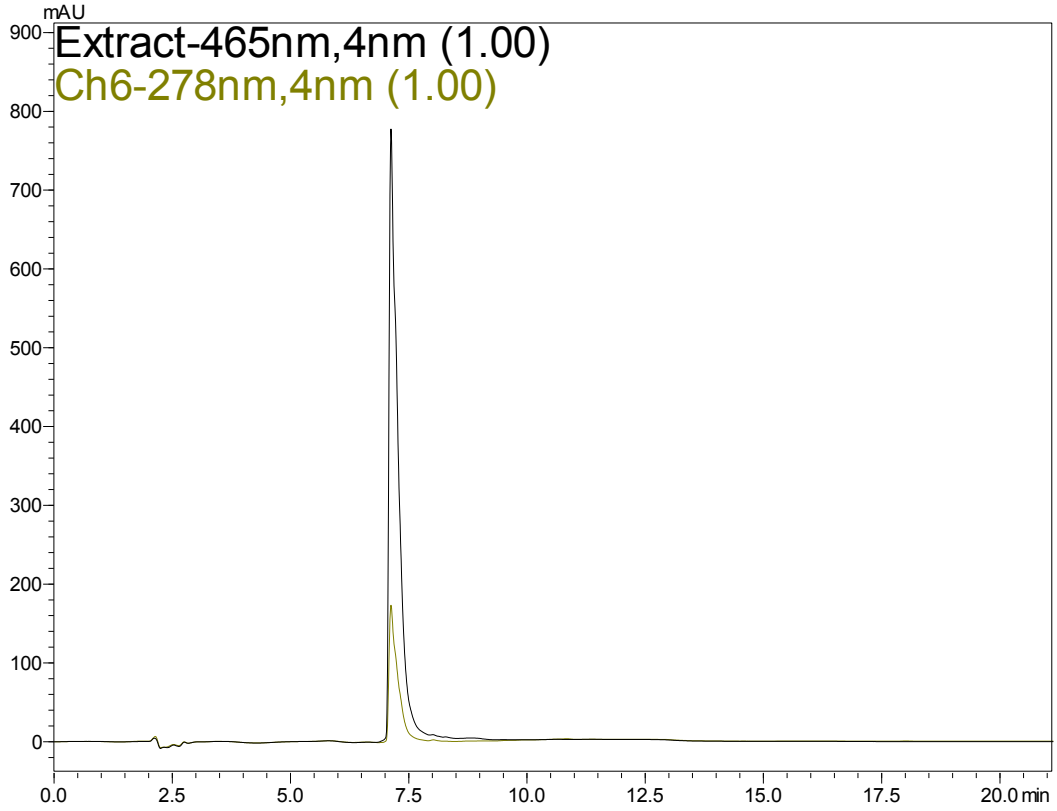
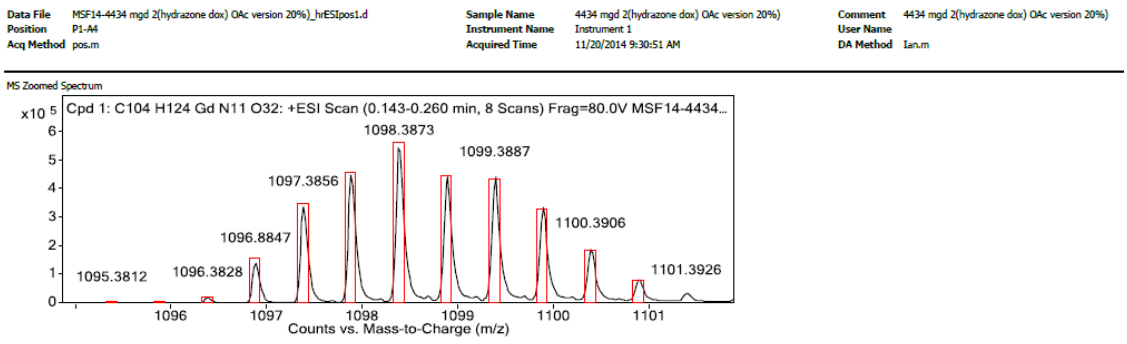


Figure S10. HPLC chromatogram of conjugate **1**.



Obs. m/z	Calc. m/z	Charge	Abund	Formula	Ion/Isotope	Tgt Mass Error (ppm)
1033.84720			604414.84			
1095.38120	1095.38000	2	2375.98	C104H124GdN11O32	M+2	-1.04
1096.88470	1096.88160	2	139661.1	C104H124GdN11O32	M+2	-2.83
1097.38560	1097.38210	2	339137.47	C104H124GdN11O32	M+2	-3.2
1097.88670	1097.88300	2	452795.54	C104H124GdN11O32	M+2	-3.35
1098.38730	1098.38350	2	558216.35	C104H124GdN11O32	M+2	-3.51
1098.88830	1098.88450	2	450221.65	C104H124GdN11O32	M+2	-3.42
1099.38870	1099.38490	2	443096.98	C104H124GdN11O32	M+2	-3.51
1099.88940	1099.88580	2	337677.83	C104H124GdN11O32	M+2	-3.28
1100.39060	1100.38710	2	191574.68	C104H124GdN11O32	M+2	-3.15

Figure S11. ESI-MS spectrum of conjugate **1**.